



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: <b>A61K 31/00</b>	A2	(11) International Publication Number: <b>WO 00/02548</b> (43) International Publication Date: <b>20 January 2000 (20.01.2000)</b>
(21) International Application Number: <b>PCT/US99/15533</b>		Published
(22) International Filing Date: <b>09 July 1999 (09.07.1999)</b>		
(30) Priority Data: <b>09/113,947 10 July 1998 (10.07.1998) US</b>		
(60) Parent Application or Grant <b>OSTEOSCREEN [/]; (O). MUNDY, Gregory, R. [/]; (O). GARRETT, I., Ross [/]; (O). ROSSINI, G. [/]; (O). MUNDY, Gregory, R. [/]; (O). GARRETT, I., Ross [/]; (O). ROSSINI, G. [/]; (O). MURASHIGE, Kate ; (O).</b>		

(54) Title: **INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE AND HAIR GROWTH**  
 (54) Titre: **INHIBITEURS D'ACTIVITE PROTEASOMIQUE POUR LA STIMULATION DE LA CROISSANCE OSSEUSE ET CAPILLAIRE**

## (57) Abstract

Compounds that inhibit the activity of NF-'kappa'B or inhibit the activity of the proteasome or both promote bone formation and hair growth and are thus useful in treating osteoporosis, bone fracture or deficiency, primary or secondary hyperparathyroidism, periodontal disease or defect, metastatic bone disease, osteolytic bone disease, post-plastic surgery, post-prosthetic joint surgery, and post-dental implantation; they also stimulate the production of hair follicles and are thus useful in stimulating hair growth, including hair density, in subject where this is desirable.

## (57) Abrégé

L'invention concerne des composés qui inhibent l'activité de NF-'kappa'B ou l'activité protéasomique ou qui stimulent à la fois la formation osseuse et la croissance capillaire. Par conséquent, ces composés sont utiles dans le traitement de l'ostéoporose, des fractures ou déficiences osseuses, de l'hyperparathyroïdie primaire ou secondaire, des parodontopathies, des troubles osseux métastatiques, des maladies ostéolytiques, des états suivant la chirurgie plastique et la chirurgie d'implantation de prothèses d'articulation ainsi que des états suivant les implantations dentaires. Ces composés sont également utiles pour stimuler la production des follicules capillaires et donc la croissance capillaire, y compris la densité capillaire, chez les sujets pour lesquels un tel traitement est souhaitable.

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(21) International Application Number: PCT/US99/15533  (22) International Filing Date: 9 July 1999 (09.07.99)		(74) Agents: MURASHIGE, Kate et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006-1888 (US).	
(30) Priority Data: 09/113,947 10 July 1998 (10.07.98)		US	(81) Designated States: AL, AM, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SD, SG, SI, SK, TR, TT, US, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US Filed on 09/113,947 (CON) 10 July 1998 (10.07.98)			
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(54) Title: INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE AND HAIR GROWTH

(57) Abstract

Compounds that inhibit the activity of NF- $\kappa$ B or inhibit the activity of the proteasome or both promote bone formation and hair growth and are thus useful in treating osteoporosis, bone fracture or deficiency, primary or secondary hyperparathyroidism, periodontal disease or defect, metastatic bone disease, osteolytic bone disease, post-plastic surgery, post-prosthetic joint surgery, and post-dental implantation; they also stimulate the production of hair follicles and are thus useful in stimulating hair growth, including hair density, in subject where this is desirable.

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**Description**

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## INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE AND HAIR GROWTH

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### Technical Field

5        The invention relates to compositions and methods for use in treating skeletal system disorders in a vertebrate at risk for bone loss, and in treating conditions that are characterized by the need for bone growth, in treating fractures, and in treating cartilage disorders. The invention also relates to enhancing hair density and growth. More specifically, the invention concerns the use of inhibitors of proteasomal activity and

15      10     inhibitors of NF- $\kappa$ B activity for these purposes.

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### Background Art

25        15     Inhibitors of proteasomal activity, and to some extent inhibitors of NF- $\kappa$ B activity, have two important physiological effects. First, they are able to enhance bone formation and are thus useful for treating various bone disorders. Second, they stimulate the production of hair follicles and are thus useful in stimulating hair growth, including hair density, in subject where this is desirable.

30

### Effect on Bone

35        20     Bone is subject to constant breakdown and resynthesis in a complex process mediated by osteoblasts, which produce new bone, and osteoclasts, which destroy bone. The activities of these cells are regulated by a large number of cytokines and growth factors, many of which have now been identified and cloned.

40        25     There is a plethora of conditions which are characterized by the need to enhance bone formation or to inhibit bone resorption. Perhaps the most obvious is the case of bone fractures, where it would be desirable to stimulate bone growth and to hasten and complete bone repair. Agents that enhance bone formation would also be useful in facial reconstruction procedures. Other bone deficit conditions include bone segmental defects, periodontal disease, metastatic bone disease, osteolytic bone disease and conditions where

45        30     connective tissue repair would be beneficial, such as healing or regeneration of cartilage defects or injury. Also of great significance is the chronic condition of osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal

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hormone status. Other conditions characterized by the need for bone growth include primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis.

There are currently no satisfactory pharmaceutical approaches to managing any of these conditions. Bone fractures are still treated exclusively using casts, braces, anchoring devices and other strictly mechanical means. Further bone deterioration associated with post-menopausal osteoporosis has been treated with estrogens or bisphosphonates, which may have drawbacks for some individuals. Although various approaches have been tried, as further discussed below, there remains a need for additions to the repertoire of agents which can be used to treat these conditions.

Treatment of bone or other skeletal disorders, such as those associated with cartilage, can be achieved either by enhancing bone formation or inhibiting bone resorption or both. A number of approaches have been suggested which relate to bone formation.

15        Bone tissue is an excellent source for factors which have the capacity for  
stimulating bone cells. Thus, extracts of bovine bone tissue obtained from  
slaughterhouses contain not only structural proteins which are responsible for maintaining  
the structural integrity of bone, but also biologically active bone growth factors which can  
stimulate bone cells to proliferate. Among these latter factors are transforming growth  
20      factor  $\beta$ , the heparin-binding growth factors (e.g., acidic and basic fibroblast growth  
factor), the insulin-like growth factors (e.g., insulin-like growth factor I and insulin-like  
growth factor II), and a recently described family of proteins called bone morphogenetic  
proteins (BMPs). All of these growth factors have effects on other types of cells, as well  
as on bone cells.

25 The BMPs are novel factors in the extended transforming growth factor  $\beta$  superfamily. Recombinant BMP2 and BMP4 can induce new bone formation when they are injected locally into the subcutaneous tissues of rats (Wozney *J Molec Reprod Dev* (1992) 32:160-67). These factors are expressed by normal osteoblasts as they differentiate, and have been shown to stimulate osteoblast differentiation and bone nodule formation *in vitro* as well as bone formation *in vivo* (Harris S. *et al. J Bone Miner Res* (1994) 9:855-63). This latter property suggests potential usefulness as therapeutic agents 30 in diseases which result in bone loss.

The cells which are responsible for forming bone are osteoblasts. As osteoblasts differentiate from precursors to mature bone-forming cells, they express and secrete a number of enzymes and structural proteins of the bone matrix, including Type-1 collagen, osteocalcin, osteopontin and alkaline phosphatase. They also synthesize a number of growth regulatory peptides which are stored in the bone matrix, and are presumably responsible for normal bone formation. These growth regulatory peptides include the BMPs (Harris S. *et al.* (1994), *supra*). In studies of primary cultures of fetal rat calvarial osteoblasts, BMPs 1, 2, 3, 4, and 6 are expressed by cultured cells prior to the formation of mineralized bone nodules (Harris S. *et al.* (1994), *supra*). Like alkaline phosphatase, osteocalcin and osteopontin, the BMPs are expressed by cultured osteoblasts as they proliferate and differentiate.

Although the BMPs are potent stimulators of bone formation *in vitro* and *in vivo*, there are disadvantages to their use as therapeutic agents to enhance bone healing. Receptors for the bone morphogenetic proteins have been identified in many tissues, and the BMPs themselves are expressed in a large variety of tissues in specific temporal and spatial patterns. This suggests that BMPs may have effects on many tissues in addition to bone, potentially limiting their usefulness as therapeutic agents when administered systemically. Moreover, since they are peptides, they would have to be administered by injection. These disadvantages impose severe limitations to the development of BMPs as therapeutic agents.

The fluorides, suggested also for this purpose, have a mode of action which may be related to tyrosine phosphorylation of growth factor receptors on osteoblasts, as described, for example, Burgener *et al. J Bone Min Res* (1995) 10:164-171, but administration of fluorides is associated with increased bone fragility, presumably due to effects on bone mineralization.

Small molecules which are able to stimulate bone formation have been disclosed in PCT applications WO98/17267 published 30 April 1998, WO97/15308 published 1 May 1997 and WO97/48694 published 24 December 1997. These agents generally comprise two aromatic systems spatially separated by a linker. In addition, PCT application WO98/25460 published 18 June 1998 discloses the use of the class of compounds known as statins in enhancing bone formation. U.S. application Serial No. 09/096,631 filed 12 June 1998 is directed to compounds for stimulating bone growth that

5 are generally isoprenoid pathway inhibitors. The contents of this application, as well as  
that of the PCT applications cited above, are incorporated herein by reference.

10 Other agents appear to operate by preventing the resorption of bone. Thus, U.S.  
15 Patent No. 5,280,040 discloses compounds described as useful in the treatment of  
5 osteoporosis. These compounds putatively achieve this result by preventing bone  
resorption.

15 Wang, G.-J. *et al.*, *J Formos Med Assoc* (1995) 94:589-592 report that certain  
lipid clearing agents, exemplified by lovastatin and bezafibrate, were able to inhibit the  
bone resorption resulting from steroid administration in rabbits. There was no effect on  
10 bone formation by these two compounds in the absence of steroid treatment. The  
mechanism of the inhibition in bone resorption observed in the presence of steroids (and  
20 the mechanism of the effect of steroid on bone *per se*) is said to be unknown.

25 An abstract entitled "Lovastatin Prevents Steroid-Induced Adipogenesis and  
Osteoporosis" by Cui, Q. *et al.* appeared in the Reports of the ASBMR 18th Annual  
15 Meeting (September 1996) *J Bone Mineral Res.* (1996) 11(S1):S510 which reports that  
lovastatin diminished triglyceride vesicles that accumulated when osteoprogenitor cells  
cloned from bone marrow stroma of chickens were treated in culture with  
30 dexamethasone. Lovastatin was reported to diminish the expression of certain mRNAs  
and to allow the cells to maintain the osteogenic phenotype after dexamethasone  
20 treatment, and chickens that had undergone bone loss in the femoral head as a result of  
dexamethasone treatment were improved by treatment with lovastatin.

35 These data are, however, contrary to reports that dexamethasone and other  
inducers, such as BMPs, induce osteoblastic differentiation and stimulate osteocalcin  
mRNA (Bellows, C.G., *et al.*, *Develop Biol* (1990) 140:132-38; Rickard, D.J., *et al.*,  
25 *Develop Biol* (1994) 161:218-28). In addition, Ducy, P. *et al.*, *Nature* (1996) 382:448-52  
40 have recently reported that osteocalcin deficient mice exhibit a phenotype marked by  
increased bone formation and bones of improved functional quality, without impairment  
of bone resorption. Ducy *et al.* state that their data suggest that osteocalcin antagonists  
45 may be of therapeutic use in conjunction with estrogen replacement therapy (for  
30 prevention or treatment of osteoporosis).

50 It has also been shown that lovastatin inhibits lipopolysaccharide-induced NF- $\kappa$ B  
activation in human mesangial cells. Guijaro, C. *et al.* *Nephrol Dial Transplant* (1996)  
11:6:990-996.

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And Its Effect on Hair Growth

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Disorders of human hair growth include male pattern baldness, alopecia areata, alopecia induced by cancer chemotherapy and hair thinning associated with aging. These 5 conditions are poorly understood, but nevertheless common and distressing, since hair is an important factor in human social and sexual communication.

15

Hair follicle regulation and growth are still not well understood, but represent dynamic processes involving proliferation, differentiation and cellular interactions during tissue morphogenesis. It is believed that hair follicles are formed only in early stages of 10 development and not replaced.

20

Hardy, M.H. *et al. Trans Genet* (1992) 8:55-61 describes evidence that bone morphogenetic proteins (BMPs), members of the TGF $\beta$  super family, are differentially expressed in hair follicles during development. Harris, S.E. *et al. J Bone Miner Res* (1994) 9:855-863 describes the effects of TGF $\beta$  on expression of BMP-2 and other 25 substances in bone cells. BMP-2 expression in mature follicles also occurs during maturation and after the period of cell proliferation (Hardy *et al.* (1992, *supra*). As noted, however, by Blessing, M. *et al. Genes and Develop* (1992) 7:204-215, the precise role 30 functional role of BMP-2 in hair follicle maturation remains unclear.

30

Approaches to treat baldness abound in the U.S. patent literature. See for example 20 U.S. Patent No. 5,767,152 (cyanocarboxylic acid derivatives), U.S. Patent No. 5,824,643 (keratinocyte growth factors) and U.S. Patent No. 5,910,497 (16-pyrazinyl-substitute-4- 35 aza-androstane 5-alpha.-reductase isozyme 1 inhibitors). There are many others.

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Gat, U. *et al. Cell* (1998) 95:605-614 has demonstrated that  $\beta$ -catenin causes adult 40 epithelial cells to create hair follicles, a surprising result in light of the known inability of mature cells to do so. B-Catenin is known to play a role in cell-cell adhesion and growth factor signal transfection. It is also known that after ubiquitination,  $\beta$ -catenin is degraded 45 by the proteasomes. Orford, K. *et al. J Biol Chem* (1997) 272:24735-24738. At least one gene associated with hair growth (or lack thereof) has also been reported. Ahmed, W. *et al. Science* (1998) 279:720-724.

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Two accepted agents currently used for the treatment of hair loss are the antihypertensive drug Minoxidil and the 5 $\alpha$ -reductase inhibitor Finasteride. Neither is entirely satisfactory. Both suffer from modest efficacy and are inconvenient to

5 administer. A specific, topically active and easy to administer compound with better efficacy than these agents would represent a marked advance.

10 Proteasomes and NF-κB

5 The present invention discloses convenient assays for compounds that will be useful in the treatment of bone disorders and in stimulating hair growth. The assays involve inhibition of the activity of the transcription factor NF-κB or of the activity of 15 proteasomal proteases, preferably proteasomal proteases. Compounds which inhibit these activities are generally useful in treating bone and hair growth disorders. Compounds that 20 inhibit the production of the transcription factor and these proteases will also be useful in the invention. Their ability to do so can be further confirmed by additional assays.

25 The proteasome is a noncompartmentalized collection of unrelated proteases which form a common architecture in which proteolytic subunits are self-assembled to form barrel-shaped complexes (for review, see Baumeister *et al.*, *Cell* (1998) 92:367-380. 30 15 The proteasome contains an array of distinct proteolytic activities inside eukaryotic cells. Compounds which inhibit proteasomal activity also reduce NF-κB activity by limiting its capacity to be translocated to the nucleus (Barnes, P.J. *et al.* *New Engl J Med* (1997) 336:1066-1071.

35 20 Disclosure of the Invention

40 The present invention adds to the repertoire of osteogenic and hair growth stimulating agents by providing drugs which would inhibit key proteins and enzymes 45 involved in proteasomal activity and which decrease the activity of the nuclear transcription factor NF-κB, and thus stimulate bone and hair growth. In accordance with 25 the present invention, we have discovered that inhibition of the functions of the proteasomal proteins and the transcription factor NF-κB in bone cells leads to increased bone growth and to hair follicle formation and stimulation. Thus, assessing a candidate compound for its ability to inhibit proteasomal proteins or NF-κB provides a useful means to identify bone and hair growth anabolic agents.

50 30 The present specification thus provides methods for identification of osteogenic compounds to stimulate bone growth and compounds that stimulate hair growth by assessing their capacity to inhibit proteasome activity or to inhibit the activity of the transcription factor NF-κB, preferably to inhibit proteasomal activity. Also useful in the

5 methods of the invention are compounds which inhibit the *in situ* production of the enzymes contained in the proteasome or inhibit the production of NF- $\kappa$ B, preferably of enzymes of the proteasomes. Once a compound found to inhibit these activities has been  
10 identified, it can be used in an additional aspect of the invention -- a method to stimulate  
5 the growth of bone or of hair by contacting suitable cells with the identified compound. The cellular contact may include *in vivo* administration and the compounds of the invention are thus useful in treating degenerative bone diseases, fractures, dental  
15 problems, baldness, alopecia and the like. These methods are performed, according to the present invention, with compounds identified as inhibitors of proteasome activity or  
10 inhibitors of the activity of transcription factor NF- $\kappa$ B, preferably inhibitors of the proteasome enzymes, or inhibitors of the production of the proteasome enzymes or of  
20 NF- $\kappa$ B, preferably of the proteasome enzymes.

Brief Description of the Drawings

25 15 Figure 1 shows a diagram of the isoprenoid pathway.

Modes of Carrying Out the Invention

30 In accordance with the present invention, there are provided methods of treating bone defects (including osteoporosis, fractures, osteolytic lesions and segmental bone  
35 20 defects) in subjects suffering therefrom said method comprising administering to said subject, in an amount sufficient to stimulate bone growth, a compound which inhibits proteasomal activity and function or the activity of the nuclear transcription factor NF- $\kappa$ B or the production of these proteins.

40 25 Also in accordance with the present invention, there are provided methods of treating disorders of hair growth. Disorders of hair growth may be the result of a defect in the ability of existing hair follicles to extrude hair, or may be the result of a deficiency in the number of hair follicles *per se*. "Stimulation of hair growth" refers to increasing the volume of hair in a particular area of a subject whether this is the result of an increased rate of growth in length and/or thickness from the same number of hair  
45 30 follicles, growth proceeding from an enhanced number of hair follicles, or both. The number of hair follicles can be enhanced by further activating existing hair follicles or by stimulating the appearance or proliferation of hair follicles in a particular region of the skin.

5                   As employed herein, the term "subject" embraces human as well as other animal species, such as, for example, canine, feline, bovine, porcine, rodent, and the like. It will be understood by the skilled practitioner that the subject is one appropriate to the desirability of stimulating bone growth or hair growth. Thus, in general, for example, 10                   5 stimulation of hair growth will be confined in most instances to animals that would appropriately exhibit such growth.

15                   As used herein, "treat" or "treatment" include a postponement of development of bone deficit symptoms and/or a reduction in the severity of such symptoms that will or are expected to develop. These terms further include ameliorating existing bone or 20                   10 cartilage deficit symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, preventing or reversing bone resorption and/or encouraging bone growth. Thus, the terms denote that a beneficial result has been conferred on a vertebrate subject with a cartilage, bone or skeletal deficit, or with the potential to develop such deficit.

25                   15 By "bone deficit" is meant an imbalance in the ratio of bone formation to bone resorption, such that, if unmodified, the subject will exhibit less bone than desirable, or the subject's bones will be less intact and coherent than desired. Bone deficit may also result from fracture, from surgical intervention or from dental or periodontal disease. By 30                   20 "cartilage defect" is meant damaged cartilage, less cartilage than desired, or cartilage that is less intact and coherent than desired. "Bone disorders" includes both bone deficits and cartilage defects.

35                   35 Representative uses of the compounds identified by the assay of the invention include: repair of bone defects and deficiencies, such as those occurring in closed, open and non-union fractures; prophylactic use in closed and open fracture reduction; 40                   25 promotion of bone healing in plastic surgery; stimulation of bone ingrowth into non-cemented prosthetic joints and dental implants; elevation of peak bone mass in pre-menopausal women; treatment of growth deficiencies; treatment of periodontal disease and defects, and other tooth repair processes; increase in bone formation during 45                   30 distraction osteogenesis; and treatment of other skeletal disorders, such as age-related osteoporosis, post-menopausal osteoporosis, glucocorticoid-induced osteoporosis or disuse osteoporosis and arthritis, or any condition that benefits from stimulation of bone formation. The compounds of the present invention can also be useful in repair of 50                   50 congenital, trauma-induced or surgical resection of bone (for instance, for cancer

5 treatment), and in cosmetic surgery. Further, the compounds of the present invention can be used for limiting or treating cartilage defects or disorders, and may be useful in wound healing or tissue repair.

10 Conditions which would be benefited by "treating" or "treatment" for stimulation 5 of hair growth include male pattern baldness, alopecia caused by chemotherapy, hair thinning resulting from aging, genetic disorders which result in deficiency of hair 15 coverage, and, in animals, providing additional protection from cold temperatures. Thus, while use in humans may be primarily of cosmetic benefit, use in animals may be therapeutic as well.

10 The compositions of the invention may be administered systemically or locally. 20 For systemic use, the compounds herein are formulated for parenteral (e.g., intravenous, subcutaneous, intramuscular, intraperitoneal, intranasal or transdermal) or enteral (e.g., oral or rectal) delivery according to conventional methods. Intravenous administration 25 can be by a series of injections or by continuous infusion over an extended period.

25 Administration by injection or other routes of discretely spaced administration can be 30 performed at intervals ranging from weekly to once to three times daily. Alternatively, the compounds disclosed herein may be administered in a cyclical manner (administration of disclosed compound; followed by no administration; followed by administration of disclosed compound, and the like). Treatment will continue until the desired outcome is 35 achieved. In general, pharmaceutical formulations will include a compound of the present invention in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water, borate-buffered saline containing trace metals or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial 40 surfaces, lubricants, fillers, stabilizers, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington's Pharmaceutical Sciences, latest edition, 45 Mack Publishing Co., Easton PA, which is incorporated herein by reference.

45 Pharmaceutical compositions for use within the present invention can be in the form of 50 sterile, non-pyrogenic liquid solutions or suspensions, coated capsules, suppositories, lyophilized powders, transdermal patches or other forms known in the art. Local administration may be by injection at the site of injury or defect, or by insertion or attachment of a solid carrier at the site, or by direct, topical application of a viscous liquid, or the like. For local administration, the delivery vehicle preferably provides a

5 matrix for the growing bone or cartilage, and more preferably is a vehicle that can be absorbed by the subject without adverse effects.

10 Delivery of compounds herein to wound sites may be enhanced by the use of controlled-release compositions, such as those described in PCT publication  
15 5 WO93/20859, which is incorporated herein by reference. Films of this type are particularly useful as coatings for prosthetic devices and surgical implants. The films may, for example, be wrapped around the outer surfaces of surgical screws, rods, pins, plates and the like. Implantable devices of this type are routinely used in orthopedic  
20 surgery. The films can also be used to coat bone filling materials, such as hydroxyapatite blocks, demineralized bone matrix plugs, collagen matrices and the like. In general, a film or device as described herein is applied to the bone at the fracture site. Application  
25 is generally by implantation into the bone or attachment to the surface using standard surgical procedures.

30 In addition to the copolymers and carriers noted above, the biodegradable films and matrices may include other active or inert components. Of particular interest are  
35 those agents that promote tissue growth or infiltration, such as growth factors. Exemplary growth factors for this purpose include epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factors (TGFs), parathyroid hormone (PTH), leukemia inhibitory factor (LIF), insulin-like  
40 growth factors (IGFs) and the like. Agents that promote bone growth, such as bone morphogenetic proteins (U.S. Patent No. 4,761,471; PCT Publication WO90/11366), osteogenin (Sampath *et al.* *Proc. Natl. Acad. Sci. USA* (1987) 84:7109-13) and NaF (Tencer *et al.* *J. Biomed. Mat. Res.* (1989) 23: 571-89) are also preferred. Biodegradable  
45 films or matrices include calcium sulfate, tricalcium phosphate, hydroxyapatite, polylactic acid, polyanhydrides, bone or dermal collagen, pure proteins, extracellular matrix components and the like and combinations thereof. Such biodegradable materials may be used in combination with non-biodegradable materials, to provide desired mechanical, cosmetic or tissue or matrix interface properties.

50 45 Alternative methods for delivery of compounds of the present invention include use of ALZET osmotic minipumps (Alza Corp., Palo Alto, CA); sustained release matrix materials such as those disclosed in Wang *et al.* (PCT Publication WO90/11366); electrically charged dextran beads, as disclosed in Bao *et al.* (PCT Publication  
55 WO92/03125); collagen-based delivery systems, for example, as disclosed in Ksander *et*

5 *al. Ann. Surg.* (1990) 211(3):288-94; methylcellulose gel systems, as disclosed in Beck *et al. J. Bone Min. Res.* (1991) 6(11):1257-65; alginate-based systems, as disclosed in  
10 Edelman *et al. Biomaterials* (1991) 12:619-26 and the like. Other methods well known in the art for sustained local delivery in bone include porous coated metal prostheses that  
5 can be impregnated and solid plastic rods with therapeutic compositions incorporated within them.

The compounds of the present invention may also be used in conjunction with agents that inhibit bone resorption. Antiresorptive agents, such as estrogen, bisphosphonates and calcitonin, are preferred for this purpose. More specifically, the compounds disclosed herein may be administered for a period of time (for instance, months to years) sufficient to obtain correction of a bone deficit condition. Once the bone deficit condition has been corrected, the vertebrate can be administered an anti-resorptive compound to maintain the corrected bone condition. Alternatively, the compounds disclosed herein may be administered with an anti-resorptive compound in a cyclical manner (administration of disclosed compound, followed by anti-resorptive, followed by disclosed compound, and the like).

In additional formulations, conventional preparations such as those described below may be used.

Aqueous suspensions may contain the active ingredient in admixture with pharmacologically acceptable excipients, comprising suspending agents, such as methyl cellulose; and wetting agents, such as lecithin, lysolecithin or long-chain fatty alcohols. The said aqueous suspensions may also contain preservatives, coloring agents, flavoring agents, sweetening agents and the like in accordance with industry standards.

Preparations for topical and local application comprise aerosol sprays, lotions, gels and ointments in pharmaceutically appropriate vehicles which may comprise lower aliphatic alcohols, polyglycols such as glycerol, polyethylene glycol, esters of fatty acids, oils and fats, and silicones. The preparations may further comprise antioxidants, such as ascorbic acid or tocopherol, and preservatives, such as p-hydroxybenzoic acid esters.

Parenteral preparations comprise particularly sterile or sterilized products.

Injectable compositions may be provided containing the active compound and any of the well known injectable carriers. These may contain salts for regulating the osmotic pressure.

5           If desired, the osteogenic agents can be incorporated into liposomes by any of the reported methods of preparing liposomes for use in treating various pathogenic conditions. The present compositions may utilize the compounds noted above 10 incorporated in liposomes in order to direct these compounds to macrophages,

15           5       monocytes, as well as other cells and tissues and organs which take up the liposomal composition. The liposome-incorporated compounds of the invention can be utilized by parenteral administration, to allow for the efficacious use of lower doses of the 20 compounds. Ligands may also be incorporated to further focus the specificity of the liposomes.

25           10       Suitable conventional methods of liposome preparation include, but are not limited to, those disclosed by Bangham, A.D. *et al. J Mol Biol* (1965) 23:238-252, Olson, 30 F. *et al. Biochim Biophys Acta* (1979) 557:9-23, Szoka, F. *et al. Proc Natl Acad Sci USA* (1978) 75:4194-4198, Kim, S. *et al. Biochim Biophys Acta* (1983) 728:339:348, and Mayer, *et al. Biochim Biophys Acta* (1986) 858:161-168.

35           15       The liposomes may be made from the present compounds in combination with any of the conventional synthetic or natural phospholipid liposome materials including phospholipids from natural sources such as egg, plant or animal sources such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine, or phosphatidylinositol and the like. Synthetic phospholipids that 40 may also be used, include, but are not limited to: dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine, and the corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. Cholesterol or other sterols, cholesterol hemisuccinate, glycolipids, cerebrosides, fatty acids, gangliosides, 45 sphingolipids, 1,2-bis(oleoyloxy)-3-(trimethyl ammonio) propane (DOTAP), N-[1-(2,3-dioleoyl) propyl-N,N,N-trimethylammonium chloride (DOTMA), and other cationic lipids may be incorporated into the liposomes, as is known to those skilled in the art. The relative amounts of phospholipid and additives used in the liposomes may be varied if desired. The preferred ranges are from about 60 to 90 mole percent of the phospholipid; 50 cholesterol, cholesterol hemisuccinate, fatty acids or cationic lipids may be used in amounts ranging from 0 to 50 mole percent. The amounts of the present compounds incorporated into the lipid layer of liposomes can be varied with the concentration of the lipids ranging from about 0.01 to about 50 mole percent.

5                   The liposomes with the above formulations may be made still more specific for  
their intended targets with the incorporation of monoclonal antibodies or other ligands  
specific for a target. For example, monoclonal antibodies to the BMP receptor may be  
10 incorporated into the liposome by linkage to phosphatidylethanolamine (PE) incorporated  
5 into the liposome by the method of Leserman, L. *et al. Nature* (1980) 288:602-604.

15                   Veterinary uses of the disclosed compounds are also contemplated, as set forth  
above. Such uses would include treatment of bone or cartilage deficits or defects  
associated with hair or fur in domestic animals, livestock and thoroughbred horses.

20                   The compounds of the present invention may be used to stimulate growth of bone-  
forming cells or their precursors, or to induce differentiation of bone-forming cell  
precursors, either *in vitro* or *ex vivo*. The compounds described herein may also modify a  
25 target tissue or organ environment, so as to attract bone-forming cells to an environment  
in need of such cells. As used herein, the term "precursor cell" refers to a cell that is  
committed to a differentiation pathway, but that generally does not express markers or  
30 function as a mature, fully differentiated cell. As used herein, the term "mesenchymal  
cells" or "mesenchymal stem cells" refers to pluripotent progenitor cells that are capable  
of dividing many times, and whose progeny will give rise to skeletal tissues, including  
35 cartilage, bone, tendon, ligament, marrow stroma and connective tissue (see A. Caplan *J.*  
*Orthop. Res.* (1991) 9:641-50). As used herein, the term "osteogenic cells" includes  
40 osteoblasts and osteoblast precursor cells. More particularly, the disclosed compounds  
are useful for stimulating a cell population containing marrow mesenchymal cells,  
thereby increasing the number of osteogenic cells in that cell population. In a preferred  
45 method, hematopoietic cells are removed from the cell population, either before or after  
stimulation with the disclosed compounds. Through practice of such methods, osteogenic  
cells may be expanded. The expanded osteogenic cells can be infused (or reinfused) into  
50 a vertebrate subject in need thereof. For instance, a subject's own mesenchymal stem  
cells can be exposed to compounds of the present invention *ex vivo*, and the resultant  
osteogenic cells could be infused or directed to a desired site within the subject, where  
further proliferation and/or differentiation of the osteogenic cells can occur without  
immunorejection. Alternatively, the cell population exposed to the disclosed compounds  
may be immortalized human fetal osteoblastic or osteogenic cells. If such cells are  
infused or implanted in a vertebrate subject, it may be advantageous to "immunoprotect"

5 these non-self cells, or to immunosuppress (preferably locally) the recipient to enhance  
transplantation and bone or cartilage repair.

10 As stated above, the compounds of the present invention may also be used to  
stimulate the growth of hair either by enhancing its rate of formation from existing  
5 follicles, stimulating inactive follicles, effecting the production of additional hair follicles  
or some combination of the foregoing, or by any other mechanism that may or may not  
presently be understood.

15 Within the present invention, an "effective amount" of a composition is that  
amount which produces a statistically significant effect. For example, an "effective  
10 amount" for therapeutic uses is the amount of the composition comprising an active  
compound herein required to provide a clinically significant increase in healing rates in  
20 fracture repair; reversal of bone loss in osteoporosis; reversal of cartilage defects or  
disorders; prevention or delay of onset of osteoporosis; stimulation and/or augmentation  
of bone formation in fracture non-unions and distraction osteogenesis; increase and/or  
25 acceleration of bone growth into prosthetic devices; and repair of dental defects. An  
"effective amount" for uses in stimulating hair growth is that amount which provides the  
desired effect in terms of length or density of hair. Such effective amounts will be  
30 determined using routine optimization techniques and are dependent on the particular  
condition to be treated, the condition of the patient, the route of administration, the  
35 formulation, and the judgment of the practitioner and other factors evident to those skilled  
in the art. The dosage required for the compounds of the invention (for example, in  
osteoporosis where an increase in bone formation is desired) is manifested as a  
40 statistically significant difference in bone mass between treatment and control groups.  
This difference in bone mass may be seen, for example, as a 5-20% or more increase in  
45 bone mass in the treatment group. Other measurements of clinically significant increases  
in healing may include, for example, tests for breaking strength and tension, breaking  
strength and torsion, 4-point bending, increased connectivity in bone biopsies and other  
biomechanical tests well known to those skilled in the art. General guidance for  
50 treatment regimens is obtained from experiments carried out in animal models of the  
disease of interest. Differences between successfully treated subjects and controls with  
regard to stimulation of hair growth can generally be ascertained by direct observation.

The dosage of the compounds of the invention will vary according to the extent  
55 and severity of the need for treatment, the activity of the administered compound, the

5 general health of the subject, and other considerations well known to the skilled artisan. Generally, they can be administered to a typical human on a daily basis as an oral dose of about 0.1 mg/kg-1000 mg/kg, and more preferably from about 1 mg/kg to about 200 mg/kg. The parenteral dose will appropriately be 20-100% of the oral dose. While oral 10 administration may be preferable in most instances where the condition is a bone deficit (for reasons of ease, patient acceptability, and the like), alternative methods of administration may be appropriate for selected compounds and selected defects or 15 diseases. While topical administration is generally preferable for stimulating hair growth, as generally only local effects are desired, systemic treatment may be preferable in some 10 instances as well.

20 Assays for Compounds Useful in the Invention

Assays for assessing the ability of a compound to inhibit proteasomal activity and for inhibitors of NF- $\kappa$ B activity are well known in the art. Two typical, but nonlimiting 25 assays are described below.

30 Assessment of Proteasomal Activity

Proteasomal activity is measured by an increase in cytoplasmic ubiquitinated protein complexes, as assessed by Western blotting using an anti-ubiquitin antibody.

35 MG-63 cells are grown in confluence in alpha MEM media and 10% fetal calf serum (FCS). Cells are then treated for 24 hours with specific compounds. Following the indicated treatments, cells are scraped with a disposable scraper, washed twice with phosphate saline solution (137 mM NaCl, 10 mM d-glucose, 4 mM KCl, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM KH<sub>2</sub>PO<sub>4</sub>), centrifuged, and the resulting pellet is suspended in the sample buffer containing 2% SDS, pH 6.75. The samples are heated and the 40 concentration of total protein calculated by means of Micro bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL/USA). The samples are diluted to obtain a final protein concentration of 2 mg/ml, supplemented with 10% 2-mercaptoethanol, 1% bromophenol blue and run on a 4-15% SDS-PAGE. Resulting gels are Western blotted with anti-ubiquitin rabbit polyclonal antibody (diluted 1:100; Sigma, St. Louis, 45 MO/USA). The samples are visualized with horse-radish peroxidase coupled anti-rabbit IgG antibodies (Amersham Corp., Arlington Heights, IL/USA) using ECL detection kits (Amersham Corp.).

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NF-κB Activity Assays

Cells are treated with different concentrations of compounds, and nuclear extracts prepared. Briefly, cells are washed with phosphate-buffered saline, and resuspended in lysis buffer (0.6% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.9, 1 mM EDTA,

10

5 0.5 mM DTT and a cocktail of protease inhibitors (Complete (TM), Boehringer Mannheim). After incubation on ice for 15 min, nuclei are collected by centrifugation. The pellet is resuspended in nuclear extraction buffer (10 mM Hepes, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM NgCl<sub>2</sub>, 0.5 mM DTT, protease inhibitors (Complete (TM), Boehringer Mannheim), 25% glycerol), and incubated at 4 degrees C for 30 min. The 10 supernatant is collected and dialyzed in a buffer containing 10 mM Tris0HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 20% glycerol. After dialysis, 20 the nuclear extract is centrifuged to remove precipitated proteins, and aliquots are stored at -70 C. Protein concentration in the nuclear extracts is measured by the method of Bradford using a dye-binding assay kit (Bio-Rad).

15

15 The probe for electrophoretic mobility shift assays is a 32P-labeled double-stranded oligonucleotide containing the consensus sequence specific for NF-κB (Promega). Nuclear extracts (5 ug) are pre-incubated in 20-ul reaction mixtures containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2.5 mM DTT, 0.5 mM EDTA, 1 mM MgCl<sub>2</sub>, 4% glycerol, and 5 ug of poly (dI-dC). After 10 min at room temperature, 10-20 20 fmol of probe is added, and incubated further for 20 min. DNA-protein complexes are separated from free oligonucleotides on a 5% polyacrylamide/0.5X TBE gel (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA). After electrophoresis, gels are dried and 35 30 autoradiographed.

30

35

Assays for Production Inhibition

40

40 Compounds which inhibit the production of the enzymes having proteasomal activity or of NF-κB can be assessed by measuring the level of production of these 45 proteins in the presence and absence of candidate compounds. The levels of production can be readily measured in *in vitro* systems using, for example, immunoassays for the 50 level of protein produced. The levels of such proteins can also be assessed by utilizing, for example, methionine labeling and size separation of proteins in the cells to be assessed. In order to effect a convenient level of protein production for measurement, it

50

55

5 is advantageous to use recombinant expression systems for the relevant enzymes or the  
NF- $\kappa$ B so that substantial amounts are produced.

10 Typical approaches to inhibiting the production of NF- $\kappa$ B or proteasome enzymes  
15 include the use of antisense technology or formation of triplexes with double-stranded  
5 forms of nucleotide sequences relevant in the expression of the genes. In addition,  
various small molecules may also inhibit this production.

15 Screening Assays - Bone

20 The osteogenic activity of the compounds used in the methods of the invention  
10 can be verified using *in vitro* screening techniques, such as the assessment of transcription  
25 of a reporter gene coupled to a bone morphogenetic protein-associated promoter or in  
alternative assays.

25 ABA Screening Assay

30 15 A rapid throughput screening test for compounds that stimulate bone formation by  
demonstration that they are capable of stimulating expression of a reporter gene linked to  
a BMP promoter (a surrogate for the production of bone morphogenetic factors that are  
35 endogenously produced) is described in U.S. Application Serial No. 08/458,434, filed 2  
June 1995, the entire contents of which are incorporated herein by reference. This assay  
20 is also described as a portion of a study of immortalized murine osteoblasts (derived from  
a mouse expressing a transgene composed of a BMP2 promoter driving expression of T-  
40 antigen) in Ghosh-Choudhery, N. *et al. Endocrinology* (1996) 137:331-39. In this study,  
the immortalized cells were stably transfected with a plasmid containing a luciferase  
50 reporter gene driven by a mouse BMP2 promoter (-2736/114 bp), and responded in a  
25 dose-dependent manner to recombinant human BMP2.

45 Briefly, the assay utilizes cells transformed permanently or transiently with  
constructs in which the promoter of a bone morphogenetic protein, specifically BMP2 or  
BMP4, is coupled to a reporter gene, typically luciferase. These transformed cells are  
then evaluated for the production of the reporter gene product; compounds that activate  
55 30 the BMP promoter will drive production of the reporter protein, which can be readily  
assayed. Many thousands of compounds have been subjected to this rapid screening  
technique, and only a very small percentage are able to elicit a level of expression of  
reporter gene 5-fold greater than that produced by vehicle. Compounds that activate the

5           BMP promoter fall into groups, where members of each group share certain structural  
characteristics not present in inactive compounds. The active compounds ("BMP  
10           promoter-active compounds" or "active compounds") are useful in promoting bone or  
cartilage growth, and thus in the treatment of vertebrates in need of bone or cartilage  
15           growth.

5           BMP promoter-active compounds can be examined in a variety of other assays  
that test specificity and toxicity. For instance, non-BMP promoters or response elements  
15           can be linked to a reporter gene and inserted into an appropriate host cell. Cytotoxicity  
can be determined by visual or microscopic examination of BMP promoter- and/or non-  
20           BMP promoter-reporter gene-containing cells, for instance. Alternatively, nucleic acid  
and/or protein synthesis by the cells can be monitored. For *in vivo* assays, tissues may be  
removed and examined visually or microscopically, and optionally examined in  
conjunction with dyes or stains that facilitate histologic examination. In assessing *in vivo*  
25           assay results, it may also be useful to examine biodistribution of the test compound, using  
conventional medicinal chemistry/animal model techniques.

Neonatal Mouse Calvaria Assay (*In vitro*)

30           An assay for bone resorption or bone formation is similar to that described by  
Gowen M. & Mundy G. *J Immunol* (1986) 136:2478-82. Briefly, four days after birth,  
35           the front and parietal bones of ICR Swiss white mouse pups are removed by  
microdissection and split along the sagittal suture. In an assay for resorption, the bones  
are incubated in BGJb medium (Irvine Scientific, Santa Ana, CA) plus 0.02% (or lower  
concentration)  $\beta$ -methylcyclodextrin, wherein the medium also contains test or control  
40           substances. The medium used when the assay is conducted to assess bone formation is  
Fitton and Jackson Modified BGJ Medium (Sigma) supplemented with 6  $\mu$ g/ml insulin,  
45           6  $\mu$ g/ml transferrin, 6 ng/ml selenous acid, calcium and phosphate concentrations of 1.25  
and 3.0 mM, respectively, and ascorbic acid to a concentration of 100  $\mu$ g/ml is added  
every two days. The incubation is conducted at 37°C in a humidified atmosphere of 5%  
CO<sub>2</sub> and 95% air for 96 hours.

50           Following this, the bones are removed from the incubation media and fixed in  
10% buffered formalin for 24-48 hours, decalcified in 14% EDTA for 1 week, processed  
through graded alcohols; and embedded in paraffin wax. Three  $\mu$ m sections of the

5 calvaria are prepared. Representative sections are selected for histomorphometric  
assessment of bone formation or bone resorption. Bone changes are measured on sections  
cut 200  $\mu$ m apart. Osteoblasts and osteoclasts are identified by their distinctive  
10 morphology.

15 5 Other auxiliary assays can be used as controls to determine non-BMP promoter-  
mediated effects of test compounds. For example, mitogenic activity can be measured  
using screening assays featuring a serum-response element (SRE) as a promoter and a  
luciferase reporter gene. More specifically, these screening assays can detect signaling  
through SRE-mediated pathways, such as the protein kinase C pathway. For instance, an  
10 osteoblast activator SRE-luciferase screen and an insulin mimetic SRE-luciferase screen  
are useful for this purpose. Similarly, test compound stimulation of cAMP response  
element (CRE)-mediated pathways can also be assayed. For instance, cells transfected  
with receptors for PTH and calcitonin (two bone-active agents) can be used in CRE-  
luciferase screens to detect elevated cAMP levels. Thus, the BMP promoter specificity of  
20 15 a test compound can be examined through use of these types of auxiliary assays.

In vivo Assay of Effects of Compounds on Murine Calvarial Bone Growth

30 Male ICR Swiss white mice, aged 4-6 weeks and weighing 13-26 gm, are  
employed, using 4-5 mice per group. The calvarial bone growth assay is performed as  
35 20 described in PCT application WO95/24211, incorporated by reference. Briefly, the test  
compound or appropriate control vehicle is injected into the subcutaneous tissue over the  
right calvaria of normal mice. Typically, the control vehicle is the vehicle in which the  
compound was solubilized, and is PBS containing 5% DMSO or is PBS containing  
40 Tween (2  $\mu$ l/10 ml). The animals are sacrificed on day 14 and bone growth measured by  
45 25 histomorphometry. Bone samples for quantitation are cleaned from adjacent tissues and  
fixed in 10% buffered formalin for 24-48 hours, decalcified in 14% EDTA for 1-3 weeks,  
processed through graded alcohols; and embedded in paraffin wax. Three to five  $\mu$ m  
sections of the calvaria are prepared, and representative sections are selected for  
histomorphometric assessment of the effects on bone formation and bone resorption.  
50 30 Sections are measured by using a camera lucida attachment to trace directly the  
microscopic image onto a digitizing plate. Bone changes are measured on sections cut  
200  $\mu$ m apart, over 4 adjacent 1x1 mm fields on both the injected and noninjected sides of

5 the calvaria. New bone is identified by its characteristic woven structure, and osteoclasts and osteoblasts are identified by their distinctive morphology. Histomorphometry software (OsteoMeasure, Osteometrics, Inc., Atlanta) is used to process digitizer input to determine cell counts and measure areas or perimeters.

10 5 Typical treatment regimens for testing utilize application of the compound to be tested over several days of repeated administration.

15 Additional *In Vivo* Assays - Bone

20 10 Lead compounds can be further tested in intact animals using an *in vivo*, dosing assay. Prototypical dosing may be accomplished by subcutaneous, intraperitoneal or oral administration, and may be performed by injection, sustained release or other delivery techniques. The time period for administration of test compound may vary (for instance, 28 days as well as 35 days may be appropriate). An exemplary, *in vivo* oral or subcutaneous dosing assay may be conducted as follows:

25 15 In a typical study, 70 three-month-old female Sprague-Dawley rats are weight-matched and divided into seven groups, with ten animals in each group. This includes a baseline control group of animals sacrificed at the initiation of the study; a control group administered vehicle only; a PBS-treated control group; and a positive control group administered a compound (non-protein or protein) known to promote bone growth. Three 30 20 dosage levels of the compound to be tested are administered to the remaining three groups.

35 25 Briefly, test compound, positive control compound, PBS, or vehicle alone is administered subcutaneously once per day for 35 days. All animals are injected with calcein nine days and two days before sacrifice (two injections of calcein administered 40 25 each designated day). Weekly body weights are determined. At the end of the 35-day cycle, the animals are weighed and bled by orbital or cardiac puncture. Serum calcium, phosphate, osteocalcin, and CBCs are determined. Both leg bones (femur and tibia) and lumbar vertebrae are removed, cleaned of adhering soft tissue, and stored in 70% ethanol 45 30 for evaluation, as performed by peripheral quantitative computed tomography (pQCT; Ferretti, J. *Bone* (1995) 17:353S-64S), dual energy X-ray absorptiometry (DEXA; Laval-Jeantet A. *et al. Calcif Tissue Intl* (1995) 56:14-18; J. Casez *et al. Bone and Mineral* (1994) 26:61-68) and/or histomorphometry. The effect of test compounds on bone 50 remodeling can thus be evaluated.

5                   Lead compounds can also be tested in acute ovariectomized animals (prevention model) using an *in vivo* dosing assay. Such assays may also include an estrogen-treated group as a control. An exemplary subcutaneous dosing assay is performed as follows:

10                  In a typical study, 80 three-month-old female Sprague-Dawley rats are weight-matched and divided into eight groups, with ten animals in each group. This includes a baseline control group of animals sacrificed at the initiation of the study; three control groups (sham ovariectomized (sham OVX) + vehicle only; ovariectomized (OVX) + vehicle only; PBS-treated OVX); and a control OVX group that is administered a compound known to promote bone growth. Three dosage levels of the compound to be tested are administered to the remaining three groups of OVX animals.

20                  Since ovariectomy (OVX) induces hyperphagia, all OVX animals are pair-fed with sham OVX animals throughout the 35 day study. Briefly, test compound, positive control compound, PBS, or vehicle alone is administered orally or subcutaneously once per day for 35 days. Alternatively, test compound can be formulated in implantable 25                  pellets that are implanted for 35 days, or may be administered orally, such as by gastric gavage. All animals, including sham OVX/vehicle and OVX/vehicle groups, are injected intraperitoneally with calcein nine days and two days before sacrifice (two injections of calcein administered each designated day, to ensure proper labeling of newly formed 30                  bone). Weekly body weights are determined. At the end of the 35-day cycle, the 20                  animals' blood and tissues are processed as described above.

35                  Lead compounds may also be tested in chronic OVX animals (treatment model). An exemplary protocol for treatment of established bone loss in ovariectomized animals that can be used to assess efficacy of anabolic agents may be performed as follows.

40                  Briefly, 80 to 100 six month old female, Sprague-Dawley rats are subjected to sham 25                  surgery (sham OVX) or ovariectomy (OVX) at time 0, and 10 rats are sacrificed to serve as baseline controls. Body weights are recorded weekly during the experiment. After 45                  approximately 6 weeks (42 days) or more of bone depletion, 10 sham OVX and 10 OVX 30                  rats are randomly selected for sacrifice as depletion period controls. Of the remaining animals, 10 sham OVX and 10 OVX rats are used as placebo-treated controls. The 50                  remaining OVX animals are treated with 3 to 5 doses of test drug for a period of 5 weeks (35 days). As a positive control, a group of OVX rats can be treated with an agent such as PTH, a known anabolic agent in this model (Kimmel *et al. Endocrinology* (1993) 132:1577-84). To determine effects on bone formation, the following procedure can be

5 followed. The femurs, tibiae and lumbar vertebrae 1 to 4 are excised and collected. The proximal left and right tibiae are used for pQCT measurements, cancellous bone mineral density (BMD) (gravimetric determination), and histology, while the midshaft of each  
10 tibiae is subjected to cortical BMD or histology. The femurs are prepared for pQCT  
5 scanning of the midshaft prior to biomechanical testing. With respect to lumbar vertebrae (LV), LV2 are processed for BMD (pQCT may also be performed); LV3 are prepared for undecalcified bone histology; and LV4 are processed for mechanical testing.

15

Assays - Hair Growth: *In Vivo* Assay of Effects of Compounds on Hair Follicles

10 Proliferation and Hair Growth

20 The assay described above to assess the effect of compounds on calvarial bone growth can also be used to assess the ability of compounds to stimulate hair growth. The test compound or appropriate control vehicle is applied to the upper and lower back of male ICR Swiss white mice either topically or by subcutaneous injection. The vehicle is  
25 selected as appropriate for the compound to be tested and for the route of administration. Optionally, the hair in the test area may be removed prior to administration. After a suitable interval, typically 7 days, the mice are anesthetized and a biopsy of the dorsal treatment area is taken using a 6 mm dermal punch. The specimens are fixed in 10%  
30 buffered formalin and imbedded in paraffin wax, and sectioned and stained to observe  
20 hair follicles. In addition, photography can be used to observe and record hair growth; typically such growth is observed after 14-18 days. After a suitable interval, typically 21  
35 days, the animals may be euthanized and the hair analyzed for fiber analysis and the tissue from the treatment area analyzed for quantitation of hair follicles.

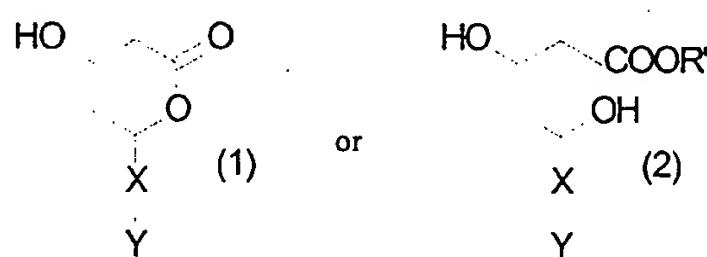
25 Nature of the Compounds Useful in the Invention

40 The compounds useful in the methods and compositions of the invention are inhibitors of proteasomal activity, of the transcription factor NF- $\kappa$ B, preferably both. Known inhibitors of these activities can be ascertained from the literature or compounds  
45 can be tested for these activities using assays known in the art. In addition, inhibitors  
30 which lower the level of effective expression of the nucleotide sequence encoding the enzymes that have proteasomal activity or of the nucleotide sequence encoding NF- $\kappa$ B can be assessed and used in the invention methods.

50

55

5        The compounds thus identified, which are used according to the method of the  
invention as it relates to treating bone defects, however, preferably do not include  
10      compounds that inhibit the isoprenoid pathway, such as the statins. A description of these  
excluded compounds can be found in WO98/25460 and in U.S. Serial No. 09/096,631,  
15      both cited above and incorporated herein by reference. For convenience, the isoprenoid  
pathway referred to is set forth herein in Figure 2. One class of compounds which are  
inhibitors are the statins which have the formula



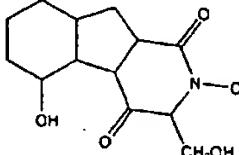
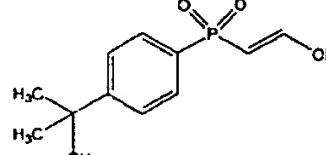
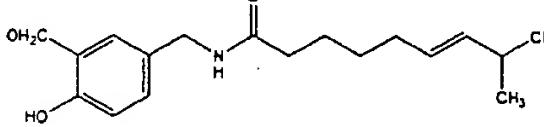
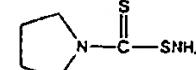
wherein X in each of formulas (1) and (2) represents a substituted or unsubstituted  
alkylene, alkenylene, or alkynylene linker of 2-6C;

10      Y represents one or more carbocyclic or heterocyclic rings wherein, when Y  
comprises two or more rings, said rings may be fused; and  
R' represents a cation, H or a substituted or unsubstituted alkyl group of 1-6C; and  
the dotted lines represent optional  $\pi$ -bonds.

15      These compounds may, however, be used in the method of the invention as it  
relates to the stimulation of hair growth.

5

Compounds known to be proteasome or NF- $\kappa$ B inhibitors include:

Proteasome Inhlbitors	
PSI	N-carbobenzoyl-Ile-Glu-(OfBu)-Ala-Leu-CHO
MG-132	N-carbobenzoyl-Leu-Leu-Leu-CHO
MG-115	N-carbobenzoyl-Leu-Leu-Nva-CHO
MG-101 or Calpain Inh I	N-Acetyl-Leu-Leu-norLeu-CHO
ALLM	N-Acetyl-Leu-Leu-Met-CHO
	N-carbobenzoyl-Gly-Pro-Phe-Leu-CHO
	N-carbobenzoyl-Gly-Pro-Ala-Phe-CHO
	N-carbobenzoyl-Leu-Leu-Phe-CHO
	N-carbobenzoyl-Leu-Ala-Leu-CHO
Gliotoxin	
SN50	NLS of NF- $\kappa$ B MW 2781
Bay 11-7082	
Capsaicin	
PDTC	

35

See, for example, Vinitsky, A. *et al. J Biol Chem* (1994) 269:29860-29866;Figueiredo-Pereira, M.E. *et al. J Neurochem* (1994) 63:1578-1581; Wojcik, C. *et al. Eur*5 *J Cell Biol* (1996) 71:311-318.

40

In the foregoing list, lactacystin is known to be an irreversible inhibitor of proteasome activity. It binds to the  $\beta$  catalytic subunit and is a specific inhibitor of the 20S proteasome. It also irreversibly inhibits NF- $\kappa$ B.

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SN50 is the NLS (nuclear localization sequence) of p50 plus the hydrophobic region of K-FGF. It inhibits the translocation of the NF- $\kappa$ B active complex to the nucleus.

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Certain peptidyl epoxy ketones such as EST are irreversible inhibitors of the proteasomes. MG-132 shows activity against the chymotryptic activity of the 20S protein

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5 without affecting its ATPase or isopeptidase activity and reversibly inhibits NF- $\kappa$ B activity. MG-115 and MG-341 show similar activities to MG-132. Various other 10 inhibitors of NF- $\kappa$ B are less active in the ABA assay. These include capsaicin, curcumin, and resiniferatoxin. Other compounds known to inhibit NF- $\kappa$ B are gliotoxin and PDTC 15 (1-pyrrolidine carbothiastic acid). Various other compounds such as BAY-11-7082 and BAY-11-7085 as well as calyculin-A inhibit phosphorylation of NF- $\kappa$ B. Calpain inhibitor inhibits calpain 1 and the proteasome; other compounds such as olomoucine and roscovitine inhibit cdk2 and/or cdk5.

An additional compound shown to be a proteasome inhibitor is pentoxyfilline 10 (PTX). Combaret, L. et al. Mol Biol Rep (1999) 26:95-101. It is active in the *in vitro* 15 *calvarial assay described above*.

20 As set forth above, in preferred embodiments of the methods of the invention, the identified compounds used in treatment of bone disorders are other than statins and other compounds that inhibit the isoprenoid pathway, typically as shown in Figure 1. In other 25 preferred embodiments, also excluded from use in the methods of treatment of bone disorders of the present invention, are compounds described in PCT applications WO98/17267, WO97/15308, and WO97/48694 cited and incorporated herein by reference hereinabove. However, the use of these compounds in the method to stimulate 30 hair growth according to the invention is not excluded.

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The following examples are intended to illustrate but not to limit the invention.

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Example 1

High Throughput Screening

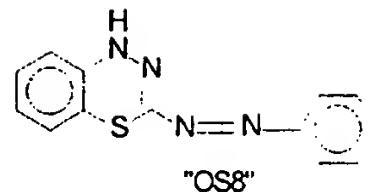
25 Thousands of compounds have been tested in the assay system set forth in U.S. 40 Serial No. 08/458,434, filed 2 June 1995, and incorporated herein by reference. Representative compounds of the invention gave positive responses, while the majority of (unrelated) compounds are inactive. In this screen, the standard positive control was the 45 compound 59-0008 (also denoted "OS8"), which is of the formula:

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In more detail, the 2T3-BMP-2-LUC cells, a stably transformed osteoblast cell line described in Ghosh-Choudhury *et al. Endocrinology* (1996) 137:331-39, referenced above, was employed. The cells were cultured using  $\alpha$ -MEM, 10% FCS with 1% penicillin/streptomycin and 1% glutamine ("plating medium"), and were split 1:5 once per week. For the assay, the cells were resuspended in a plating medium containing 4% FCS, plated in microtiter plates at a concentration of  $5 \times 10^3$  cells (in 50  $\mu$ l)/well, and incubated for 24 hours at 37°C in 5% CO<sub>2</sub>. To initiate the assay, 50  $\mu$ l of the test compound or the control in DMSO was added at 2X concentration to each well, so that the final volume was 100  $\mu$ l. The final serum concentration was 2% FCS, and the final DMSO concentration was 1%. Compound 59-0008 (10  $\mu$ M) was used as a positive control.

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The treated cells were incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The medium was then removed, and the cells were rinsed three times with PBS. After removal of excess PBS, 25  $\mu$ l of 1X cell culture lysing reagent (Promega #E153A) was added to each well and incubated for at least ten minutes. Optionally, the plates/samples could be frozen at this point. To each well was added 50  $\mu$ l of luciferase substrate (Promega #E152A; 10 ml Promega luciferase assay buffer per 7 mg Promega luciferase assay substrate). Luminescence was measured on an automated 96-well luminometer, and was expressed as either picograms of luciferase activity per well or as picograms of luciferase activity per microgram of protein.

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In this assay, compound 59-0008 (3-phenylazo-1H-4,1,2-benzothiadiazine) exhibits a pattern of reactivity which is maximal at a concentration of approximately 3-10  $\mu$ M. Accordingly, other tested compounds can be evaluated at various concentrations, and the results compared to the results obtained for 59-0008 at 10  $\mu$ M (which value would be normalized to 100). Alternatively, the reactivity of a compound to be tested can be compared directly to a negative control containing no compound.

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The control compound 59-0328, which is simvastatin, gives a good response. The known proteasome inhibitors MG-132 and MG-115 also show high activity; MG-132 is

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5 effective at lower concentrations. Positive responses are also obtained using lactacystin. However, gliotoxin, olomoucine, roscovitine, SN50, PDTC, and capsaicin do not give promising responses.

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Example 2In vitro Bone Formation

15 Selected compounds and appropriate controls were assayed *in vitro* (*ex vivo*) for bone formation activity (described above in "Techniques for Neonatal Mouse Calvaria Assay (*in vitro*)). Histomorphometrical assessments of *ex vivo* calvaria were carried out

10 using an OsteoMetrics bone morphometry measurement program, according to the manufacturer's instructions. Measurements were determined using either a 10- or 20-fold

20 objective with a standard point counting eyepiece graticule.

25 New bone formation was determined (using a 10X objective) by measuring the new bone area formed in one field in 3 representative sections of each bone (4 bones per group). Each measurement was carried out  $\frac{1}{2}$  field distance from the end of the suture. Both total bone and old bone area were measured. Data were expressed as new bone area in  $\mu\text{m}^2$ .

30 The results in Example 1 were somewhat imperfectly correlated with the results in this assay. The control compound, simvastatin showed new bone formation in this assay

35 as did MG-132 and lactacystin. MG-115 also showed positive results although less dramatic than those of simvastatin. However, gliotoxin, which appeared negative in the ABA assay of Example 1 did demonstrate the ability to stimulate bone growth. The remaining compounds, olomoucine, roscovitine, SN50, PDTC and capsaicin appeared negative in this assay.

40 25 Osteoblast numbers are determined by point counting. The number of osteoblast cells lining the bone surface on both sides of the bone are counted in one field using a 20X objective. Data are expressed as osteoblast numbers/mm of bone surface.

45 30 Alkaline phosphatase activity is measured in the conditioned media of the murine organ cultures, using the method described by Majeska, R.J. *et al. Exp Cell Res* (1978) 111:465-465. Conditioned media are incubated at 37°C for 20 minutes with phosphatase substrate 104 (Sigma) and the reaction stopped with 2 ml of 0.1 M NaOH. Alkaline phosphatase activity is calculated by measuring cleaved substrate at an optical density of

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5        410 nm in a Beckman dual beam spectrophotometer from the OD410 and corrected for  
protein concentration.

10        PSI and MG-132 and control compounds/factors bFGF and BMP-2, and a vehicle  
control were tested in this assay and the calvaria were analyzed histomorphometrically, as  
5        described above. Increase in bone area as a function of concentration; the increase in  
osteoblasts and the enhancement of alkaline phosphatase activity for PSI were measured.

15        The data show that PSI is as good as, or better than, BMP-2 and bFGF (two "gold  
standard" agents for bone growth; see Wozney J. *Molec Reprod Dev* (1992) 32:160-67;  
WO95/24211) for inducing bone formation.

20        An additional experiment, pentoxyfilline (PTX) was tested in the foregoing assay.  
It exhibited the ability to enhance new bone formation in concentrations as low as 0.1  $\mu$ m.  
20        At a concentration of 10  $\mu$ m, PTX appeared to enhance the new bone area over control by  
over 100%; at 100  $\mu$ m, the increase was approximately three (3) times that of control.

25        Example 3

15        In vivo Calvarial Bone Growth Data

30        PSI and MG-132 were assayed *in vivo* according to the procedure described  
previously (see "*In vivo Assay of Effects of Compounds on Murine Calvarial Bone  
Growth*", *supra*). As a control, simvastatin provided a 1.5 fold increase in the number of  
osteoblasts.

35        In one experiment, vehicle control, bFGF and varying doses of PSI were tested in  
the *in vivo* calvarial bone growth assay. The results are reported as a measurement of  
total bone area, % increase in area over vehicle control, and % increase in new bone  
width as shown below.

Compound	Total Bone Area ( $\mu$ m <sup>2</sup> )	% Increase* in Bone Area Compared with Control	% Increase† in New Bone Width
Control	0.64 $\pm$ 0.03		
0.1 mg/kg/day	0.74 $\pm$ 0.02	21.7 $\pm$ 3.5	
1 mg/kg/day	0.83 $\pm$ 0.02	35.4 $\pm$ 3.4	19.9 $\pm$ 2.0
5 mg/kg/day	0.79 $\pm$ 0.03	32.1 $\pm$ 5.6	19.9 $\pm$ 4.4

40        \*p < 0.05        †p < 0.001

45        25        In addition, histological examination showed confirmation of bone growth both  
when 5 mg/kg/day of PSI was used and 1 mg/kg/day was used.

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Example 4Summary of Effects on Bone Formation

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The table below summarizes the results obtained for compounds tested in the various assays set forth above. It is seen that compounds that are proteasome inhibitors also enhance bone formation. In the compounds tested in this table, however, compounds which are known to be inhibitors only of NF- $\kappa$ B but which fail to inhibit proteasomal activity, do not enhance luciferase activity (indicative of BMP-2 promoter activity) in the high through-put assay, nor do they enhance bone formation in the calvarial assay *in vitro*, to as great an extent as do proteasome inhibitors.

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Compounds useful in the invention include:

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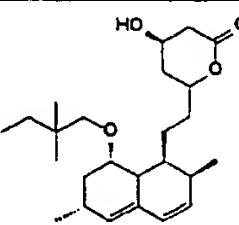
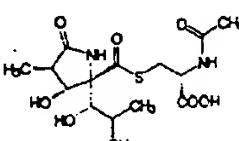
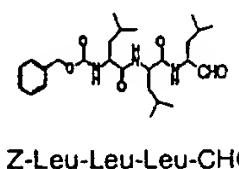
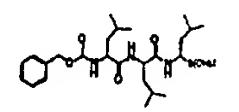
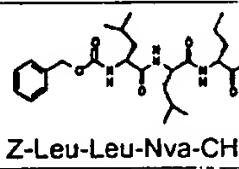
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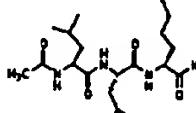
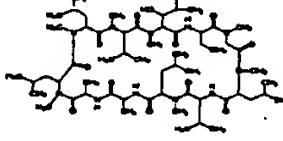
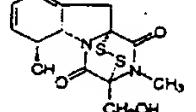
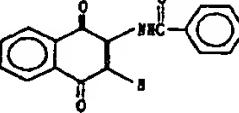
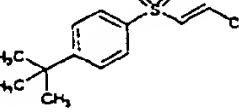
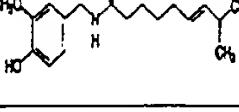
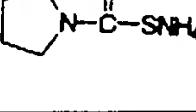
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Compound	Structure	Luciferase Activity (ED <sub>50</sub> - $\mu$ M)	Bone Formation (ED <sub>50</sub> - $\mu$ M)	Proteasome Activity (ED <sub>50</sub> - $\mu$ M)			
Simvastatin		↑↑	0.2	↑↑	0.2	↓	-
Lactacystin		↑↑	1	↑↑	1	↓↓	1.5
PS1	Z-Lie-Glu(OtBu)-Ala-Leu-CHO	↑↑	0.05	↑↑	0.03	↓↓	0.035
MG132		↑↑	0.25	↑↑	0.5	↓↓	0.3
MG262		↑↑	0.1	↑↑	0.1	↓↓	0.07
MG115		↑↑	2	↑↑	1	↓↓	1

5	Compound	Structure	Luciferase Activity (ED <sub>50</sub> -μM)	Bone Formation (ED <sub>50</sub> -μM)	Proteasome Activity (ED <sub>50</sub> -μM)	
10	ALLN	 N-Acetyl-Leu-Leu-Nle-CHO	↑↑	10	-	↓ 1.5
15	Cyclosporin A		-	↑↑	10	↓ 1.0
20	Gliotoxin		-	↑↑	10	-
25	SN50	NLS of NF-KB MW 2781	-	-	-	-
30	ALLM	N-Acetyl-Leu-Leu-Meth-CHO	-	-	↓ 4	-
35	PPM-18		-	-	-	-
40	Bay 11-7082		-	-	-	-
45	Capsaicin		-	-	↓ 30	-
50	PDTC		-	-	-	-

5

Example 5Effect of PSI and Other Proteasome Inhibitors on Hair Follicle Production

The *in vivo* bone calvarial growth assay of Example 3 was modified to observe the number of hair follicles in treated mice. In initial observations, PSI (5mg/kg/day) was 10 injected three times a day for 5 days over the calvaria of Swiss ICR mice as described 5 above. Sixteen days later the mice were sacrificed. Histology of the calvaria revealed a strikingly large increase in the number of hair follicles in those mice treated with PSI 15 versus control mice. In addition to PSI, MG132 (10mg/kg), MG115 (10mg/kg) and lactacysin administered in the same way also stimulated an increase in the number of hair 20 follicles.

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Example 6Stimulation of Hair Growth

Male Swiss ICR mice were first treated to remove hair from the scalp and dorsal 25 regions as follows. Paraffin wax was liquefied by heating to 55°C and the liquefied wax then applied by brush to the scalp and/or back (under anesthesia). The wax was allowed 30 to solidify and then removed. The day following hair stripping, PSI (1 mg/kg/day) was injected subcutaneously three times a day for five days into the scalp and dorsal region. On day 7 a dermal punch biopsy was taken; histology revealed a large increase in the 35 number of hair follicles in mice administered PSI versus control mice. By day 18, it was observable that the treated mice had a hair growth rate greater than that of the mice in the control group.

The mice were sacrificed on day 21 and histology was performed on the dermis of 40 the scalp and of the dorsal region. In the treated mice, mature hair follicles in numbers 45 much greater than in controls had migrated to the lower region of the dermis. Upon closer examination, it was observed that mice that had received only vehicle had quiescent hair follicles. When treated with PSI such follicles were stimulated to differentiate into mature hair follicles and to migrate to the lower region of the dermis.

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Example 7Topical Administration

PSI was prepared as a topical formulation, where the vehicle was 50% propylene 50 glycol, 30% ethanol, 20% deionized water, at 0.1% concentration of PSI. The solution

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5 was applied 3 times a day for 5 days. The mice in a treated group were observed as compared to controls similarly treated with vehicle alone. The results at day 16 showed stimulation of hair growth relative to the controls.

10 In addition to stimulating hair growth, PSI was able to thicken both the hair and  
5 the hair shaft. PSI increases hair count when the follicle area is greater than 0.01 mm<sup>2</sup>.  
When the protocol above was repeated using a 0.5% solution of PSI in groups containing  
15 5 mice each, the number of hairs per 0.8 mm<sup>2</sup> was 60 in the treated mice versus about 10  
in the control group. The percentage of follicle area in a region of about 0.8 mm<sup>2</sup> was  
about 30% as an average in the treated group as compared to 15% as an average in the  
10 control group.

20 Example 8  
Dose Requirements

25 In order to determine the minimal effective dosage of PSI, when used topically, a  
15 dose response curve for PSI was prepared. All experiments were preformed according to  
current good laboratory practice regulations (21CFR58). The mice were divided into 7  
groups, 10 mice each, wherein one group was control treated only with vehicle and  
30 groups 1-6 with a series of increasing concentrations of PSI in a vehicle comprising 50%  
propylene glycol, 30% ethanol, 20% deionized water. The concentrations were 0.006%,  
20 0.012%, 0.025%, 0.05%, 0.1% and 0.5%.

35 The mice were anesthetized (50 µl Mouse Cocktail containing 3 ml ketamine, 2  
ml small animal rompum, 5 ml NaCl), identified by ear punch code, weighed and the hair  
on the dorsal side removed by waxing as described in Example 6. After waxing, the  
40 animals were photographed. On the following day (day 1), 100 µl of PSI at the above  
25 concentrations in vehicle was brushed onto the area of removed hair. A similar  
application of PSI solution was performed daily for an additional 4 days.

45 On Day 7 mice were anesthetized and a biopsy of the dorsal treatment area taken  
using a 6 mm dermal punch; the specimens were fixed in 10% buffered formalin and  
embedded in paraffin wax. Sections were cut using a standard microtome.

50 30 Mice were monitored daily for signs of hair growth, and any hair growth was  
recorded by photography. On day 21 animals were euthanized (75mg/kg body weight  
phenobarbital, IP injection), 2 cm hair samples were taken for optical based fiber analysis,  
and the remaining dorsal treatment area was fixed in 10% buffered formalin for further

5 histological analysis. Analysis included quantification of hair thickness and  
10 quantification of mature hair follicles. Results were expressed as the mean = +/- the  
standard error of the mean. Data were analyzed by repeated measures of analysis of  
variance followed by the Tukey-Kramer post test P values of <0.05 were considered  
5 significant.

The results indicate that the minimal effective dose of PSI is 0.5% applied 1 time a day for 4 days; additional experiments showed that 0.1% of PSI applied topically 3 times a day for 5 days was also effective.

Gross observation of mice receiving an effective dose indicated an enhanced rate  
10 of hair growth, a thickening of hair diameter, increase in sheath diameter, and  
differentiation of quiescent hair follicles into more mature forms.

20 The contents of all documents cited above are expressly incorporated herein to the extent required to understand the invention.

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**Claims**

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Claims

1. A method to enhance bone formation or to treat pathological dental conditions or to treat degenerative joint conditions in a vertebrate animal which method  
10 comprises administering to a vertebrate subject in need of such treatment an effective amount of a compound that inhibits the activity of NF- $\kappa$ B or that inhibits proteasomal activity or that inhibits production of proteasome proteins.

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10 2. The method of claim 1 wherein said compound inhibits proteasomal activity or that inhibits production of proteasomal proteins.

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3. The method of claim 1 wherein said compound does not inhibit the isoprenoid pathway.

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15 4. The method of claim 1 wherein said compound is lactacystin, a peptidyl aldehyde, or PTX.

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20 5. The method of claim 1 wherein said subject is characterized by a condition selected from the group consisting of osteoporosis, bone fracture or deficiency, primary or secondary hyperparathyroidism, periodontal disease or defect, metastatic bone disease, osteolytic bone disease, post-plastic surgery, post-prosthetic joint surgery, and post-dental implantation.

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25 6. The method of claim 1 which further comprises administering to said subject one or more agents that promote bone growth or that inhibit bone resorption.

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30 7. The method of claim 6 wherein said agents are selected from the group consisting of bone morphogenetic factors, anti-resorptive agents, osteogenic factors, cartilage-derived morphogenetic proteins, growth hormones, estrogens, bisphosphonates, statins and differentiating factors.

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50 8. A method to treat a mammalian subject for a condition benefited by stimulating hair growth which method comprises administering to said mammalian

5 subject in need of such treatment an effective amount of a compound that inhibits the activity of NF- $\kappa$ B or that inhibits proteasomal activity or that inhibits production of these proteins.

10 5 9. The method of claim 8 wherein said compound inhibits proteasomal activity or that inhibits production of proteasome proteins.

15 10. The method of claim 9 wherein said compound is lactacystin or a peptidyl aldehyde.

20 10 11. A pharmaceutical composition for treating bone disorders, dental pathological conditions or degenerative joint conditions which composition comprises a compound that inhibits the activity of NF- $\kappa$ B or that inhibits proteasomal activity or that inhibits production of these proteins.

25 15 12. The pharmaceutical composition of claim 11 wherein said compound inhibits proteasomal activity or that inhibits production of proteasomal proteins.

30 13. The pharmaceutical composition of claim 11 wherein said compound does 20 not inhibit the isoprenoid pathway.

35 14. The pharmaceutical composition of claim 11 wherein said compound is lactacystin, a peptidyl aldehyde, or PTX.

40 25 15. The pharmaceutical composition of claim 11 wherein said compound does 40 not inhibit the isoprenoid pathway.

45 16. A pharmaceutical composition for treating for a condition benefited by stimulating hair growth which composition comprises a compound that inhibits the 30 activity of NF- $\kappa$ B or that inhibits proteasomal activity or that inhibits production of these proteins.

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5                   17. The pharmaceutical composition of claim 13 wherein said compound is lactacystin or a peptidyl aldehyde.

10                   18. A method to identify a compound which enhances bone growth or

5                   stimulates hair growth which method comprises subjecting said compound to an assay for determining its ability to inhibit NF- $\kappa$ B activity, whereby a compound which inhibits the activity of NF- $\kappa$ B is identified as a compound which enhances bone growth; or

15                   subjecting said compound to an assay for determining its ability to inhibit the production of NF- $\kappa$ B, whereby a compound which inhibits the production of NF- $\kappa$ B is

10                   identified as a compound which enhances bone growth; or

20                   subjecting a candidate compound to an assay to assess its ability to inhibit proteasomal activity, whereby a compound which inhibits proteasomal activity is identified as a compound that enhances bone growth; or

25                   subjecting a candidate compound to an assay to assess its ability to inhibit the production of enzymes with proteasomal activity, whereby a compound which inhibits the production of enzymes with proteasomal activity is identified as a compound that enhances bone growth.

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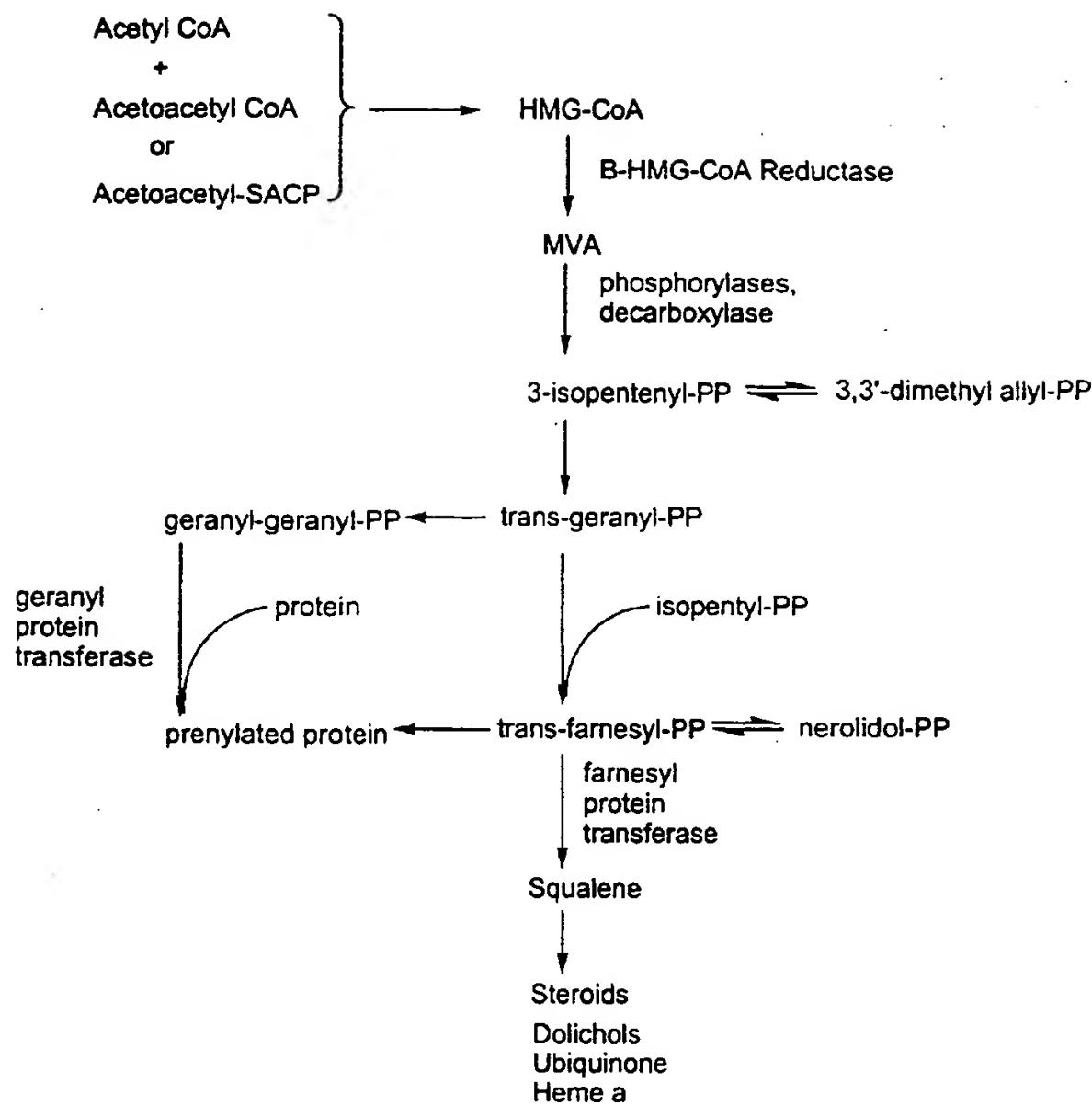


Figure 1